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Simple System for the Preliminary Evaluation of Infectivity and Pathogenesis of Insect Virus in a Nontarget Estuarine Shrimp

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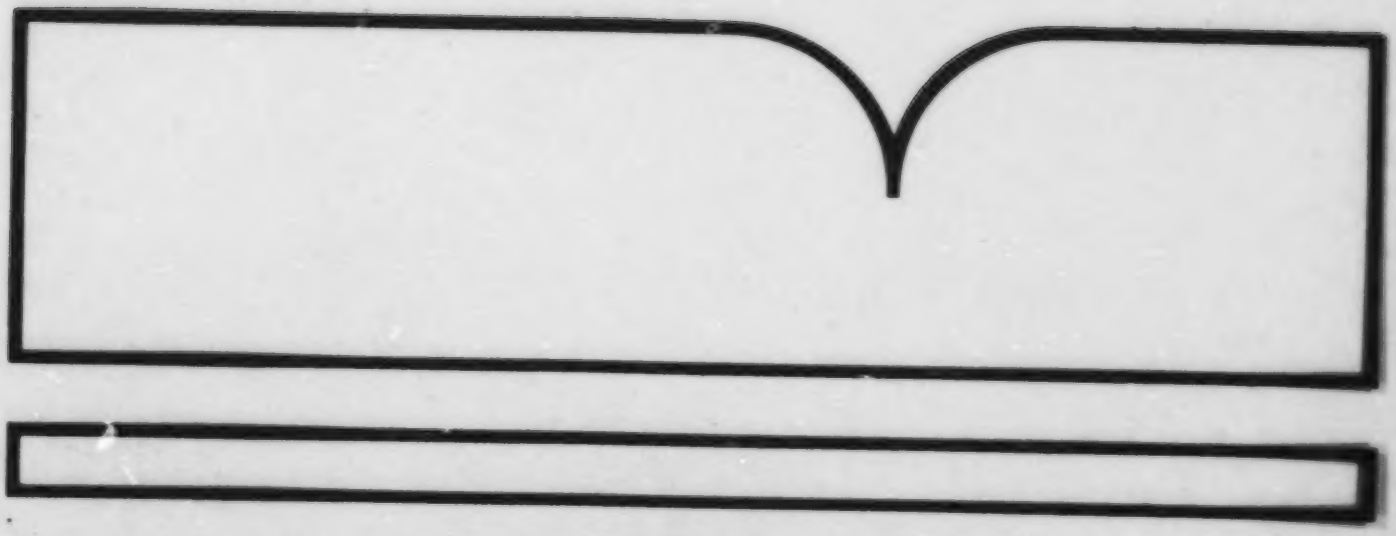

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16. ABSTRACT

Biological control agents (biorationals) are increasingly important in pest control concepts. Certain insect viruses, particularly the baculoviruses (nuclear polyhedrosis viruses), are considered to have potential as biological pesticides, and could be used widely in the environment. Therefore, test animals must be selected and methods developed to evaluate the safety of biorationals to non-target species.

A simple laboratory system has been designed and tested to determine risks of infectivity and pathogenicity of an insect Baculovirus, originally isolated from the Alfalfa looper Autographa californica, to a non-target arthropod, the grass shrimp, Palaemonetes vulgaris, by dietary exposure. This laboratory method also permits testing of other microbial biorationals against non-target aquatic species, and provides an inexpensive, procedure of safety testing.

Results from this study indicated that histopathological, ultrastructural and serological methods used provided no evidence that experimental exposure to the virus caused viral infection or related pathogenicity in the grass shrimp.

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JOURNAL ARTICLE

A Simple System for the Preliminary Evaluation of Infectivity and Pathogenesis of Insect Virus in a Nontarget Estuarine Shrimp

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Biological control agents (biorationals) are increasingly important in pest control concepts. Certain insect viruses, particularly the baculoviruses (nuclear polyhedrosis viruses), are considered to have potential as biological pesticides and could be used widely in the environment. Therefore, test animals must be selected and methods and laboratory systems developed to evaluate the safety of these agents to nontarget species. A simple laboratory system has been designed and used to determine risks of infectivity and pathogenicity of an insect *Baculovirus*, originally isolated from the Alfalfa looper, *Autographa californica*, to a nontarget arthropod, the grass shrimp, *Palaemonetes vulgaris*, by dietary exposure. This laboratory method also permits evaluation of other microbial biorationals against nontarget aquatic species, and provides an inexpensive standardized procedure of safety testing. Results from this study indicated that histopathological, ultrastructural, and serological methods used provided no evidence that experimental exposure to the virus in our test system caused viral infection or related pathogenicity in the grass shrimp.

KEY WORDS: *Baculovirus; Autographa californica* NPV; biorationals; biological controls; *Palaemonetes vulgaris;* nontarget host; test system.

INTRODUCTION

Biological control agents (biorationals) are of increasing importance in integrated pest control concepts. Certain insect viruses, particularly the baculoviruses, are considered to have potential as microbial pesticides in their target insect hosts (Falcon, 1969).

Because certain of these agents may be developed and produced as controls for insects, and as such possibly used widely in the environment, methods must be developed to evaluate their safety for nontarget species (Korp, 1975; Environmental Protection Agency, 1982), particularly those nontarget species phyletically close to the target species.

The present work is concerned with the results of the design, development, and laboratory testing of a simple system for determining risks of infectivity and pathogenicity of a select baculovirus by dietary exposure to a nontarget arthropod, the grass shrimp (Crustacea:Decapoda). The grass shrimp, *Palaemonetes vulgaris* (Fig. 1), is a representative estuarine arthropod that could conceivably be exposed to viral biorationals used in agricultural applications. Though many different aquatic species could have been selected for use in the development of a laboratory system, we

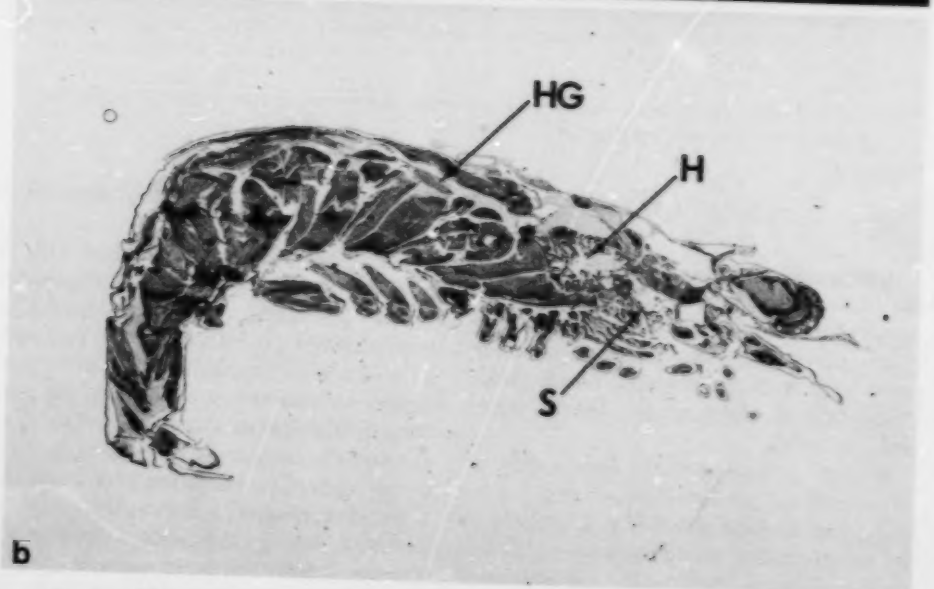

FIG. 1. (a) Photograph of a grass shrimp, *Palaemonetes vulgaris*. Average length: 22–42 mm. (b) parasagittal section of *P. vulgaris*, showing target organ, the hepatopancreas, and stomach and hindgut (H, S, HG). Harris hematoxylin and eosin, 7 μm.

chose the grass shrimp because of its phylogeny, availability, laboratory adaptability, widespread estuarine and geographical distribution, and ecological role in the aquatic environment (Couch, 1975a, b).

The purpose of evaluating the grass shrimp laboratory test system is to provide a method whereby microbial biorationals such as viruses, bacteria, fungi, and protozoa could be exposed to select, nontarget aquatic species utilizing a standard procedure for safety testing. We believe that the methods reported here are adaptable for the laboratory evaluation of the risks of many of these agents to a representative nontarget aquatic arthropod.

MATERIALS AND METHODS

Test Animals and Test Conditions

Approximately 300 adult grass shrimp were collected from grass beds in Santa Rosa Sound, Florida, and held in a large outdoor aquarium for 30 days. At the end of this time a routine histological baseline sample using 50 of the animals was taken to determine the presence of any preexisting inclusion body virus or other pathogen in the shrimp, with negative results. Tissues examined included stomach, midgut, hindgut, hepatopancreas, muscle, and gonads (Fig. 1b).

Two 76-liter glass tanks were filled to a 3-cm depth with clean oyster shell over filtration systems, equipped with additional sidemounted filters, and aerators, then filled with water piped directly in from Santa Rosa Sound (25 ppt). During the test, evaporation loss was replaced with deionized water. Thus, the animal maintenance system was completely static (Fig. 2).

One hundred and twenty shrimp were taken from the acclimated source group; they were selected for size similarity and nongravid status only. Two shrimp were placed in each of 60 holding cups constructed of a Nitex screen (450 mesh) cylinder cemented to glass Petri dish bottoms with silicone adhesive to form a chamber 15 cm high and 6 cm in diameter (Fig. 2). Thirty holding cups were then placed on a glass tray in the experimental aquarium, and thirty placed in a second control aquarium. Water level in the aquaria was maintained at approximately 8 or 9 cm below the top of the holding cups (Fig. 2).

Exposure of test animals via feeding appeared to be the most efficient, natural and certain method to ensure internal exposure to the virus short of injection. During a second period of acclimation (30 days) in the test aquarium, different feeding regimens were attempted in order to maximize survival and ensure statistical validity of the test. Inadequate food supply caused cannibalism, whereas excessive food caused bacterial contamination which also endangered shrimp survival. We found that virus exposure probably was most assured if shrimp were fed virus-contaminated food pellets twice a week (one pellet per cup, per feeding) and excess food was removed from the holding cups the next day. The feeding, cleaning, and sampling chronology is given in Figure 3.

When animals died or were eaten before the test began, they were replaced from the original source group. During the test, the shrimp were checked twice daily to ensure that weakened or dead specimens were recovered in time to make histological examination possible. All animals that died before test termination were fixed in a combination fixative (Hayat, 1981) for light and electron microscopy, labeled, and put aside to be examined with those shrimp killed at the experiment's end. Prior to termination, no casualties were replaced in the system.

Virus: Preparation and Feeding

A pure preparation of freeze-dried *Autographa californica* nuclear polyhedrosis virus, a pathogenic baculovirus produced in the alternate host insect, *Spodoptera exigua*, was introduced to the shrimp via food pellets (virus prepared by Dr. G. Tompkins). Standard Tetra flake food was ground to a fine powder in separate coffee grinders, manipulated, and pelleted with separate sets of utensils. One set of equipment was used to prepare virus-containing pellets,

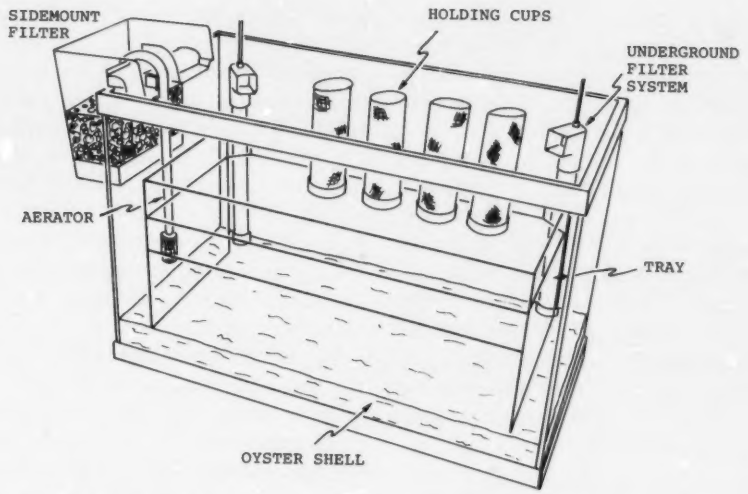

FIG. 2. Recirculating static test system used in this study. Note cups for holding two shrimp each. The actual aquarium could be increased in size to hold larger numbers of animals, if desired.

the other virus-free (control) pellets. Food was formed into tablets that weighed approximately 0.2 g in a commercial pellet maker with a 6.5-mm die.

Shrimp were fed twice weekly with one pellet per holding cup. Control animals received virus-free pellets; experimental shrimp were fed pellets that contained 15 $\times$ 10^6 PIBs (polyhedral inclusion bodies)/pellet. This concentration far exceeded the amount needed to cause significant mortality in the virus host insect (more than 3 $\times$ 10^3 the LD_{90} concentration).

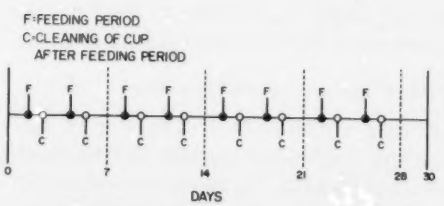

FIG. 3. Chronological scheme of the feeding, cleaning, and sampling pattern utilized during this experiment. Note that removal of excess food quickly followed each feeding period in order to prevent undesirable microbial growth in the sea water. At day 30, all surviving animals were sacrificed and fixed for histology or frozen for serology. F = feeding period; C = cleaning of cup after feeding period.

During feeding the sidemounted filters were turned off to limit virus circulation during peak feeding. Filters were left running the rest of the time because of crowded tank conditions and because feeding tended to slack off several hours after the pellets were introduced.

The day after feeding, all holding cups were removed to plastic trays, excess food was pipetted out into a large glass flask, and the cups were returned to their tanks. Virus-contaminated refuse was decontaminated by adding full-strength Clorox to the glass flask, plastic tray, and utensils. The refuse containers were immersed in this solution for several hours, then emptied and rinsed thoroughly. Waste from the control aquarium was merely washed down with drain water.

Bioassay of Food

Commercial food pellets used to feed the shrimp were bioassayed by incorporating 2 g of the shrimp food into semisynthetic insect medium and placing one neonatant cabbage looper larva into each assay cup (Thomas et al., 1972). For the control food (commercial preparation) and food that

contained the LD_{50} dose of polyhedra, 36 larvae were used in each of two replicates; thus, 72 *Trichoplusia ni* larvae were exposed to the commercial food and 72 were exposed to polyhedra-dosed commercial food. There were also 72 *T. ni* and *S. exigua* serving as controls with no commercial food or polyhedra added to the semisynthetic insect diet.

Serological Testing

After 30 days exposure, the test was terminated, and all surviving animals were killed and processed. Frozen specimens of 18 exposed and 18 control shrimp (both whole body and visceral masses) were prepared for serological testing.

Homogenization of samples. Each sample was put in a 36-ml centrifuge tube with 15 ml of Tris–barbital buffer, *p*H 8.6, kept cool in an ice bath, homogenized by sonification in a microanalyzer for approximately 30 sec, then centrifuged at 5000*g* for 5 min. The supernatant was decanted (Supernatant A), frozen, and used as an antigen source. The pellet was suspended in 1 ml of distilled water. Slides made from the suspension were examined microscopically for polyhedra. Then 2 ml was added to the suspension, shaken lightly for 1 hr and 45 min at 26°C, then centrifuged at 5000*g* for 10 min and the supernatant decanted. This supernate was frozen for later use.

Antisera were prepared against whole *A. californica* NPV polyhedra, *A. californica* NPV polyhedral protein, and *T. ni* NPV polyhedral protein according to the method of Tompkins (1979). The antisera were ammonium sulfate precipitated and stored at −20°C. Immunodiffusion was performed against these three antisera and polyhedral protein of *A. californica* MNPV produced in beet armyworm, using 1% Bio-Rad agarose in Tris–barbital buffer, *p*H 8.6. The slides were incubated for 72 hr at 4°C in a moist chamber, overlaid with 1.0 M NaCl for 3 hr, rinsed with distilled water for 10 min, dried, stained in a solution of 2% Coomassie brilliant blue R-250, destained, and photographed.

Immunoelectrophoresis was performed in 10 × 10-cm glass slides that contained a medium of 15 ml of 1% Bio-Rad agarose in Tris–barbital buffer, *p*H 8.6. Wells were punched in the medium and appropriate antigens added and allowed to electrophorese in Tris–barbiturate buffer at 8.3 V/cm, 9°C, for 1 hr and 45 min. After electrophoresis, appropriate antisera were added and the slides incubated for 73 hr at 4°C in a moist chamber, then washed, stained, and destained as previously described with the immunodiffusion slides.

All test and control supernates from the homogenized shrimp, and all supernates from the pellets after addition of 0.1 M Na_2CO_3–0.1 M NaCl, were immunodiffused and immunoelectrophoresed using antisera against *T. ni* virions, polyhedral protein, and/or antisera to *A. californica* polyhedral protein.

Histology

Thirty-eight shrimp (19 exposed, 19 control) were sacrificed at the end of the test and preserved in Davidson's fixative (Shaw and Battle, 1957). Complete visceral masses were removed and processed for light microscopy. Following embedding in paraffin, 7-μm longitudinal sections were stained with either routine Harris hematoxylin and eosin or with a modified bromophenol blue technique (Johnson, 1980), then examined for inclusion bodies, with major emphasis on the nuclei of hepatopancreas and midgut cells.

Electron Microscopy

Hepatopancreas specimens reserved for electron microscopy were fixed in either gluteraldehyde or combination fixative, postfixed in osmium tetroxide, and processed and embedded in Epon. Silver sections, cut on an ultramicrotome, were stained with lead citrate. Nuclei and cytoplasm of hepatopancreas cells from random samples were searched at 2500× to 5000× in a Zeiss EM10 electron microscope. All fields on each grid were examined for presence or absence of inclusion

body, virus, or cytopathic effect. Control shrimp samples were examined similarly.

RESULTS

Bioassay

There was less than 5% mortality in the *T. ni* larvae or *S. exigua* larvae fed synthetic shrimp diet without virus. Mortality was 47% among *T. ni* larvae fed synthetic shrimp diet containing an LD_{50} dose of *A. californica* virus, and 54.6% among S. *exigua* so fed.

Gross Observations

Control and experimental shrimp, after the introduction of the virus, did not exhibit differences in feeding behavior, equilibrium, or activity. Mortality during acclimation to the holding cups was 35–40%. During the test, cumulative mortality (for 30 days) was 30% among control animals and 28% among experimental animals.

Serology

Immunodiffusion patterns showed similar precipitin bands between antisera against *A. californica* polyhedral protein and antisera against *T. ni* polyhedral protein to *A. californica* polyhedral protein. Although whole *A. californica* polyhedral protein gave no visible reaction in the immunodiffusion, it did give similar precipitin bands in the immunoelectrophoresis.

Since the antisera against *T. ni* polyhedrin and *A. californica* polyhedrin gave the most prominent bands, either of these two antisera was used for the rest of the immunodiffusion and immunoelectrophoresis tests.

No serological evidence of presence of the polyhedral protein or virion protein was found by immunodiffusion or immunoelectrophoresis utilizing exposed shrimp tissues. Complement fixation tests were also negative.

Histology

Light microscopical examination revealed no difference between tissues of control shrimp and those exposed to the viral PIBs. No evidence of inclusion bodies or any other pathogenic response was seen in the hepatopancreas or midgut. Nor was there indication of altered nuclei or cytoplasmic change usually indicative of viral infection.

Electron Microscopy

Careful examination of representative fields in ultrathin sections of hepatopancreas revealed no indications of viral infection or cytopathologic activity characteristic of viral infections.

DISCUSSION

We have designed a simple, inexpensive experimental system by which microbial pesticides can be evaluated for safety considerations in an estuarine shrimp. Our objectives included selection of a representative animal (nontarget aquatic arthropod) and development of an inexpensive test system that required minimum space, maintenance, and safety controls. These objectives are met with the grass shrimp, *P. vulgaris*, which can be maintained in large numbers in a static system. Few other aquatic arthropods meet the criteria of availability, adaptability, and ease of maintenance.

In this study, *P. vulgaris* survived the entire test period at an acceptable level to determine possible effects of the agent (72% survival for experimental shrimp, 70% for control shrimp). Results indicated that the concomitant mortality was not of viral origin. Death of control and experimental shrimp was probably related to injury accrued in capture and acclimation and/or antagonistic behavior (cannibalism) during

the experiment. Whenever arthropods are housed together, some mortality may result from these factors. Histopathological, ultrastructural, and serological methods used provided no evidence that experimental exposure resulted in viral infection or related pathogenicity.

It should be reiterated that we provided a dosage of viral PIBs to the shrimp many times that required to cause an LD_{50} in the host target insect species, to enhance the possibility of infection. We believe that exposure of this virus to the shrimp is most effective via the diet, as all individuals were observed to feed on the virus-contaminated food pellets. Bioassay controls (see Results) proved that the target insect species was infected after feeding on commercial shrimp diet contaminated with viral PIBs (insect LD_{50} equivalents). Other methods of exposure (through the ambient medium alone, or by injection, for example) seemed inadequate or unnecessarily complicated unless they provide basis for the measurement of endpoints other than those measured herein.

This simple, economical system could be utilized with other biorational agents that can be administered as discrete infectious particles, i.e., protozoans (spores), fungi (spores), and bacteria (spores or vegetative cells). Other test aquatic species could also be utilized in this system, providing their maximum size and physiologic requirements permitted their maintenance.

Test animals exposed in this type of system may provide tissues from which other endpoints (for infectivity, pathogenicity, or toxicity) could be measured.

REFERENCES

COUCH, J. A. 1975a. Discussion. *In* "Baculoviruses for Insect Pest Control: Safety Considerations" (Max Summers, ed.), pp. 60–62. Amer. Soc. Microbiol., Washington, D.C.

COUCH, J. A. 1975b. Discussion. *In* "Baculoviruses for Insect Pest Control: Safety Considerations" (Max Summers, ed.), pp. 113–114. Amer. Soc. Microbiol., Washington, D.C.

Environmental Protection Agency. 1982. "Pesticide Assessment Guidelines," Subdivision M, "Biorational Pesticides." Office of Pesticides and Toxic Substances, Washington, D.C.

FALCON, L. A. 1969. Microbial control as a tool in integrated control programs. *In* "Biological Control" (E. C. B. Huffaker, ed.), pp. 346–364. Plenum, New York.

HAYAT, M. A. 1981. "Principles and Techniques of Electron Microscopy: Biological Applications," Vol. I, p. 128. Univ. Park Press, Baltimore, Md.

JOHNSON, P. 1980. "Histology of the Blue Crab, *Callinectes sapidus:* A Model for the Decapoda," pp. 395, 396, 407. Praeger, New York.

KORP, H. 1975. Introductory Remarks. *In* "Baculoviruses for Insect Pest Control: Safety Considerations" (Max Summers, ed.), p. 3. Amer. Soc. Microbiol., Washington, D.C.

SHAW, B. L., AND BATTLE, H. I. 1957. The gross and microscopic anatomy of the digestive tract of the oyster *Crassostrea virginica* (Gmelin). *Canad. J. Zool.*, **35**, 325–347.

THOMAS, E. D., REICHELDERFER, C., AND HEIMPEL, A. 1972. Accumulation and persistence of a nuclear polyhedrosis virus of the cabbage looper in the field. *J. Invertebr. Pathol.* **20**, 157–164.

TOMPKINS, G. T. 1979. "Characterization of Multiple Embedded Nuclear Polyhedrosis Viruses Isolated from the Cabbage Looper, *Trichoplusiana,* and the Alfalfa Looper, *Autographa californica.*" Ph.D. thesis, University of Maryland.